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(71) Applicant *(for all designated States except US)*: MINISTERO UNIVERSITA' RICERCA SCIENTIFICA E TECNOLOGICA [IT/IT]; Piazzale Kennedy, 20, I-00144 Rome EUR (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MELE, Antonio [IT/IT]; Via Magnani, 16, I-51016 Montecatini Terme (IT). DE SANTIS, Rita [IT/IT]; Via Don Luigi Sturzo, 18/D, I-00040 Pomezia (IT). FERRER MARSAL, Cristina [IT/IT]; Via del Teatro Romano, 13, I-00042 Anzio (IT). ANASTASI, Anna, Maria [IT/IT]; Via Enrico Medi, 14, I-00149 Rome (IT). DI MASSIMO, Anna, Maria [IT/IT]; Via della Vittoria, 19, I-67068 Scurcola Marsicana (IT). COLNAGHI, Maria, Ines [IT/IT]; Strada Al Lago, 12, I-20090 Milano San Felice (IT).

(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi S.r.l., Viale Bianca Maria, 33, I-20122 Milano (IT).

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(54) Title: MURINE/HUMAN CHIMERIC MONOCLONAL ANTIBODY OR A FRAGMENT THEREOF SPECIFIC FOR THE EGF RECEPTOR

(57) Abstract

A murine/human chimeric monoclonal antibody (termed chMint5), obtained by the murine monoclonal chMint5, which is specific for the EGF-receptor, is described. Particularly, the nucleotide sequences and the amino acid sequences encoded by them of the variable regions (VH and VL) of such chMint5 antibody, are disclosed.

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MURINE/HUMAN CHIMERIC MONOCLONAL ANTIBODY OR A FRAGMENT THEREOF SPECIFIC FOR THE EGF RECEPTOR.

Field of the Invention

This invention relates to a murine/human chimeric monoclonal antibody, termed chMint5, which is specific for the EGF-receptor. Particularly, the nucleotide sequences and the amino acid sequences encoded by them of the variable regions (VH and VL) of such chimeric antibody, chMint5, are disclosed.

Anterior Art

Mint5 is a monoclonal antibody specific for the EGF-receptor (EGF-R), obtained by immunization of mice with A431 cell line.

The major feature of chMint5 is its capability of discriminating between cells having normal levels of EGF-R expression and cells having high levels of such expression. Mint5 has got an inhibitory effect on growth of cells having high levels of EGF-R expression in experiments either in vivo or in vitro, but it has got no effect on cells having normal levels of receptor expression.

Once bound to A431 cells, Mint5 is efficiently internalized. Moreover, capability of inhibiting growth of tumor cells which were transplanted in athymic mice was shown.

The cell line of hybridoma producing Mint5 antibody was filed in 1993, the seventh of september, by DSM (Deutsches Sammlung von Mikroorganismen und Zellkulturen GmbH) with number ACC2150.

The variable domains are determining for the antigenic identification of the tumor cell. However, the murine origin of such antibody limits its use in clinical therapy owing to the immune response of the host

receiver.

Summary of the Invention

Authors of this invention prepared a novel chimeric monoclonal antibody, termed chMint5, which could show a lower immnunogenicity when administered to humans.

Particularly, this invention relates to a chimeric monoclonal chMint5 antibody or to a fragment thereof comprising a certain amino acid sequence of the variable regions of the heavy chain (VH) and light chain (VL) of the monoclonal antibody obtained by the hybridoma deposited as DSM ACC2150 (murine Mint5).

This invention relates, therefore, to nucleotide sequences coding the variable regions of the heavy chains (VH) and light chains (VL) of chMint5 antibody, to the correspondent amino acid sequences and it also relates to chMint5 antibody and to the fragments thereof comprising said sequences of the variable regions, and comprising whole constant regions or parts thereof of the heavy and light chains obtained from human immunoglobulins.

chMint5 antibody can be used alone or combined to cytotoxic molecules according to the present invention in the antitumor therapy of patients affected by tumors characterized by a high level of EGF-R expression in a diagnostic assay specific for the determination of the EGF-R. This diagnostic method comprises a ligand or a conjugate (e.g. another antibody), eventually labeled or bound to a chromogen, which recognize the variable regions of said chMint5.

Description of Figures and List of the Sequences

- Figure 1 shows a schematic illustration of the cDNA amplification of the heavy chain (H) of chMint5.

- Figure 2 shows a schematic illustration of the procedure of chMint5 expression in CHO cells.

- Figures 3A and 3B show FACS analysis (Fluorescence Activated Cell Sorter) of chMint5 on EGF-R^+ and EGF-R^- cell lines. The cells ($1X10^6$) were incubated into ice for 30 minutes, with chMint5 or PBS/1%FCS (negative control). The cells were then washed with PBS/1%FCS and incubated with FITC-conjugated anti-human-IgG1 murine antibody (fluorescein isothiocyanate; Sigma), then washed again and analyzed by using a FACS (FACSort, Becton-Dickinson).

- Figure 4a shows the affinity constant of murine Mint5 bound on A431 cells. The characteristics are the following:

Molecular weight: 155000; specific activity (Ci/g):10.7; counter efficiency: 0.84; volume of the sample: 0.1 ml; non-specific calculations: 1061.

- Figure 4b shows the affinity constant of chMint5 bound on A431 cells.

The characteristics are the following:

Molecular weight: 155000; specific activity (Ci/g); 19.56; counter efficiency: 0.84; volume of the sample: 0.1 ml; non-specific calculations: 0.

In Figures 4a and 4b, the affinity constant was determined by means of SAB-method as described in Antoni G. and Mariani M., 1985, J. Immunol. Methods, 83:61-68.

- SEQ ID NO:1 sequence shows the nucleotide and amino acid sequences of the variable region of the chMint5 antibody heavy chain. The nucleotide sequences of 8 codons at 5'-end and of 11 codons at 3'-end were established by primers used in the cDNAs amplification by means

of PCR. The primers were selected according to works by Orlandi et al., PNAS, 86:3833-3837 (1989). Sequences indicated as CDR1, CDR2 and CDR3 (CDR= Complementarity Determinant Region) refer to hypervariable regions.

- SEQ ID NO:2 sequence shows the amino acid sequence (deduced from the nucleotide sequence indicated in SEQ ID NO:1) of the variable region of the chMint5 antibody heavy chain.

- SEQ ID NO:3 sequence shows a N-terminal fragment of the amino acid sequence of the variable region of the heavy chain. Said sequence was determined by means of Edman degradation. Only No. 1,3,5 and 6 amino acids differ from the deduced amino acid chain indicated in SEQ ID NO:2. Amino acids indicated as Xaa were not correctly defined. As these didn't influence the identification of the variable region, the antibody was not subjected to further analysis of the proteic sequence.

- SEQ ID NO:4 sequence shows the nucleotide and the amino acid sequences of the variable region of the chMint5 antibody light chain. The nucleotide sequence of eight codons (24 nucleotides) at 5'- and 3'- ends were established by primers used in the cDNAs amplification by means of PCR. Sequences indicated as CDR1, CDR2 and CDR3 refer to hypervariable regions.

- SEQ ID NO:5 sequence shows the amino acid sequence (deduced by the nucleotide sequence indicated in SEQ ID NO:4) of the variable region of the chMint5 antibody light chain.

- SEQ ID NO:6 sequence shows a N-terminal fragment of the amino acid sequence of the variable region of the chMint5 antibody light chain. Said sequence was determined by means of Edman degradation. Only No. 3

and 8 amino acids differ from the deduced amino acid sequence indicated in SEQ ID NO:5. Amino acids indicated as Xaa were not correctly defined. As these didn't influence the identification of the variable region, the antibody was not subjected to further analysis of the proteic sequence.

- SEQ ID NO:7 shows the nucleotide sequence of a 5' primer specific for the leader sequence of the light and heavy chains for use in PCR.
- SEQ ID NO:8 shows the nucleotide sequence of CKhu primer specific for the constant region of the light chain for use in PCR.
- SEQ ID NO:9 shows the nucleotide sequence of the antisensus VH1FOR primer specific for the sequence of a fragment of the constant region of the heavy chain for use in PCR.
- SEQ ID NO:10 shows the nucleotide sequence of the CHhu primer specific for the sequence at the 3'-end of the constant region fragment of the heavy chain for use in PCR.

Detailed Description of the Invention

Murine/human chimeric monoclonal chMint5 antibody was obtained by fusion of the genes of the variable regions of the light and heavy chains of murine Mint5 antibody at the nucleotide sequences coding for human immunoglobulin constant regions.

Particularly, for the chMint5 preparation, the VH and VL nucleotide sequences were bound to the nucleotide sequences of human C-gamma1 and CK genes.

The realization of this invention will be clarified on the basis of the following examples.

Example 1

Preparation of chMint5 antibody

Genes for chimeric (murine/human) light and heavy chMint5 chains were essentially prepared as described by Orlandi et al., 1989, PNAS, 86:3833-3837.

Total RNA from Mint5 hybridomas (DSM ACC2150) was isolated and VH and VL genes were obtained by amplification by means of PCR, using consensus primers. The amplified VH and VL genes were cloned in M13-derived vehicles (in M13VHPCR1 vehicle and in M13VKPCR1 vehicle respectively, courteously given by Dr.G.Winter) containing the promoter and the leader sequence of the immunoglobulin genes, and then sequenced.

The VH fragment gene was directly cloned in M13VHPCR1 vehicle and sequenced (Sequenase^{R} Version 2.0 U.S.B., USA). Instead, the VL gene (then indicated as VK, too) was at first introduced in M13mp19 vehicle in order to remove the two inside BamHI and PvuII restriction sites, by means of site-directed mutagenesis (Oligonucleotide-Directed In vitro mutagenesis System Version 2. Amersham International plc, Little Chalfont, UK). These restriction sites would have hinder the VK insertion into M13VKPCR1 vehicle and the subcloning in the vehicle of expression for the chimeric light chain (alfa-Lys17 vehicle). The thus modified VK gene was then cloned in M13VKPCR1 vehicle and at last sequenced.

For the preparation of the chimeric heavy and light chains genes, the VH and VK genes of Mint5 were inserted in alfa-Lys30 and alfa-Lys17 vehicles, respectively (courteously given by Dr.G.Winter).

Alfa-Lys30 plasmid contains the human Cgamma1 chain gene and gpt gene (xanthine-guanine phosphoribosyl transferase) as selection marker.

pMRS18 vehicle of expression was therefore prepared. Said vehicle of

expression was obtained by cloning HindIII-BamHI fragment, obtained from M13VHPCR1 vehicle, which contains the promoter, the leader sequence and the Mint5 VH region in alfa-Lys30 vehicle, upstream from the human constant region gene.

Alfa-Lys17 plasmid contains the human CK chain gene and the gene for resistance to hygromicine as selection marker.

pMRS17 vehicle of expression was therefore prepared. Said vehicle of expression was obtained by cloning HindIII-BamHI fragment, obtained from M13VKPCR1 vehicle which contains the promoter, the leader sequence and the Mint5 VK region in alfa-Lys17 vehicle, upstream from the human constant region gene.

In order to obtain the stable expression of Mint5 antibody, non-secernent-immunoglobulin murine myeloma NS0/1 cells were cotransfected with pMRS18 and pMRS17 vehicles by means of electroporation (Potter et al., 1984, PNAS, 81:7161-7165). The stable transfectans were analyzed and selected according to their mycophenolic acid resistance and hygromicine resistance and according to the chimeric antibody production by means of ELISA.

The transfectomas in NS0 cells showed chMint5 productivity of 1-5 ng/10^6 cells/24 hours. These productivity levels, although they didn't allow the preparatory purification of the chimeric antibody, showed that chMint5 genes are correctly translated by transfected NS0/1 cells which can be then utilized for the isolation of cDNAs of the chMint5 heavy and light chains in order to achieve a better expression.

Example 2

Isolation of cDNAs of the chMint5 heavy and light chains and expression thereof in CHO cells.

From 10^8 cells selected from the mostly productive transfected NSO/1 clones, total RNA was extracted and the genes of the chimeric chains were amplified by reverse transcription followed by PCR, as indicated in the schematic illustration of Figure 1.

The primers utilized in PCR for the amplification of the chMint5 cDNAs are indicated in the following Table.

DNA fragment	5'-end	3'-end
A	leader primer (SEQ ID NO:7)	CKhu (SEQ ID NO:8)
B	leader primer (SEQ ID NO:7)	VH1FOR(1)
C	antisensus VH1FOR (SEQ ID NO:9)	CKhu (SEQ ID NO:10)

A = cDNA of the light chain

B = cDNA of the fragment of the variable region of the heavy chain

C = cDNA of the fragment of the constant region of the heavy chain

(1)VH1FOR primer was described in Orlandi et al., (as above).

PCR was performed by using leader primers complementary to the 5'-leader region of the heavy and light chains by inserting a XbaI site. For human heavy and light constant 3' regions, CHhu and CKhu primers respectively were used, primers which introduce a SmaI site.

The cDNA of the chimeric heavy chain was amplified in two phases (according to the scheme of Figure 1):

a) the leader and variable regions were amplified by using a leader

primer at the 5'-end and VH1FOR primer at the 3'-end; the constant region was amplified by using, at the 5'-end, antisensus VH1FOR primer (complementary to VH1FOR primer), and CHhu primer at 3'-end;

b) products of such amplifications were amplified once again, all together in order to achieve the whole heavy chain.

Two pSV2-derived vehicles, termed pMRS81 and pMRS71 were used to express the genes of the chMint5 heavy and light chains respectively, in CHO cells.

pMRS81 was obtained from pMCMVbeta51-2. This vehicle was digested with AccI and HindIII, thus eliminating the immediate early promoter of murine Cytomegalovirus. At AccI-HindIII sites the immediate early promoter of human cytomegalovirus was cloned, with AccI-HindIII end (promoter which was disclosed in Int. Appln. No. WO95/11982 filed on October 24, 1994.

In this way, pUthCMVbeta vehicle was obtained. In this vehicle, the 2.25 kbp fragment with BamHI-end, which was obtained from pMSG vehicle (Pharmacia) and containing a transcription unit for gpt gene, was cloned. In pMRS81 vehicle the two eukaryotic transcription units were oriented in the same direction.

pMRS71 was obtained from pMCMVbeta51-2 vehicle. For such purpose, pMCMVbeta51-2 vehicle was linearized with BamHI and treated with alkaline fosphatase from intestinal calf mucosa (CIP); in the thus treated vehicle, the transcription VIDHFR unit was inserted. In pMRS71 vehicle, the two eukaryotic transcription units are oriented in the same direction. pMCMVbeta51-2 vehicle and the transcription VIDHFR unit are mentioned in Int. Appln. No. WO95/11982 filed on October 24, 1994.

pMRS81 is, therefore, a plasmid containing the early promoter of human Cytomegalovirus, the polyadenylation signal of the 1-betaglobin of rabbit and the gtp gene as selection marker. cDNA of the chMint5 heavy chain was cloned in XbaI-SmaI cloning site as XbaI-blunt fragment owing to the presence of an inside SmaI site in the human constant region. The thus obtained vehicle was termed pMRS89.

cDNA of the chimeric light chain, amplified from transfected NSO/1 cells, was cloned as a XbaI-SmaI fragment in pMRS71 plasmid containing the early promoter of murine Cytomegalovirus, the polyadenylation signal of 1-betaglobin of rabbit and the gene of dihydrofolate reductase (dhfr) as amplificable selection marker. The thus obtained vehicle was termed pMRS95.

CHO dhfr$^-$ cell line was cotransfected with pMRS89 and pMRS95 vehicles (see Figure 2) by lipofection (Felgner P. et al., 1987, PNAS, 84, 7413-7417). The transfectants were selected according to the dhfr$^+$/gpt double resistance phenotype. The single clones were moved to 24-well plates and after one week of growth, the supernatants of culture were tested for production of chMint5 by ELISA.

Such transfectant clones secreted amounts of chMint5 from 50 to 100 ng/10^6 cells/24 hours. After amplification with methotrexate (MTX) (up to 600 nM), the productivity appeared increased to 1-2 microg/10^6 cells/24 hours.

Example 3

Characterization of chMint5

The Mint5 antibody was purified from supernatants of cultures of a CHO transfectant single clone, by means of affinity chromatography on A Protein-Sepharose.

The binding activity of the chimeric antibody was analyzed by means of flow cytometry. A431 cells of human epidermoid carcinoma and human Jurkat T EGF-R negative cells were used in the assay. The results, illustrated in Figure 3, show that chMint5 specifically recognizes the A431 cells (EGF-R^+), but it doesn't bind the Jurkat T cells (EGF-R^-). The binding affinity constants for murine and chimeric antibodies were determined by means of the titration curves of the monoclonal antibody, obtained by IRMA on A431 target cells.

Figures 4a and 4b show a Kaff value (affinity constant) of $6.76X10^9$ M^{-1} for chMint5, whereas Mint5 value was $2.05X10^{10}$ M^{-1}, thus indicating that chMint5 substantially keeps the binding affinity properties of the murine Mint5.

The monoclonal chMint5 antibody showed to keep the specificity and the binding affinity properties of the murine Mint5 antibody. chMint5 also shows a greater utilization in the human tumor therapy. chMint5 antibody can be used for the preparation of a pharmaceutical composition, useful in human therapy, comprising a pharmaceutically acceptable carrier and/or excipient.

chMint5 can be also used in the preparation of immunotoxins or immunocytokines.

Such immunotoxins or immunocytokines can be obtained by means of chemical conjugation or by recombination of genes which correspond to chMint5 or to parts of it and by recombination of genes coding for the toxins or the cytokines which will be in the immune-complex.

Further this invention relates to the use of such immunotoxins or immunocytokines for the preparation of pharmaceutical compositions useful in the human antitumor therapy, combined to a pharmaceutical

acceptable carrier and/or excipient.

chMint5 can be also used in a diagnostic test specific for the EGF-R. Such diagnostic method comprises the chMint5 antibody and a ligand or a conjugate (for example another antibody), eventually labeled or bound to a chromogen, which recognize the variable regions of said chMint5. Preferably, such ligand or conjugate will comprise a sequence complementary to at least one of the sequences indicated in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 or to fragments thereof.

This invention hence relates to the use of chMint5 in diagnostic test,too, as described above.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MINISTERO UNIVERSITA' RICERCA SCIENTIFICA E TECNOLOGICA
(B) STREET: Piazzale Kennedy, 20
(C) CITY: Rome EUR
(E) COUNTRY: ITALY
(F) POSTAL CODE (ZIP): 00144

(ii) TITLE OF THE INVENTION: Murine/human chimeric monoclonal antibody or a fragment thereof specific for the EGF receptor

(iii) NUMBER OF SEQUENCE: 10

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatibile
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Versione #1.25 (EPO)

(v) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IT FI 95 A 000036
(B) FILING DATE: 01-MAR-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..360

(ix) FEATURE:
(A) NAME/KEY: CDR1
(B) LOCATION: 91..105

(ix) FEATURE:
(A) NAME/KEY: CDR2
(B) LOCATION: 150..198

(ix) FEATURE:
(A) NAME/KEY: CDR3
(B) LOCATION: 295..327

(ix) FEATURE:
(B) LOCATION: 1..24
(D) OTHER INFORMATION: note= "This sequence was imposed by the primer used in the cDNA amplification by means of PCR"

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(B) LOCATION: 328..360
(D) OTHER INFORMATION: note= "This sequence was imposed by the primer used in the cDNA amplification by means of PCR"

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TAC ATG TAT TGG GTT CGC CAG ACT CCA GAG AAG AGG CTG GAG TGG GTC       144
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        35                  40                  45

GCA TAC ATT AGT AAT GGT GGT GGT AGC ACC TAT TAT CCA GAC ACT GTA       192
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    50                  55                  60

AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC       240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65                  70                  75                  80

CTG CAA ATG AGC CGT CTG AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT       288
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GCA AAC TCT CTC TAC TTT GAT TTC GAC GAT CTC TCT TAC TGG GGC CAA       336
Ala Asn Ser Leu Tyr Phe Asp Phe Asp Asp Leu Ser Tyr Trp Gly Gln
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) CHAIN: singlE
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE: Xaa rapresents an amino acid not exactly characterized

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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20 25 30

Tyr Met Xaa Xaa Val
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) ANTI-SENSE: NO

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(B) LOCATION: 1..324

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(A) NAME/KEY: CDR1
(B) LOCATION: 70..102

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(B) LOCATION: 148..168
(D) OTHER INFORMATION: CDR2

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(B) LOCATION: 265..291

(ix) FEATURE:
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /note= "This sequence was imposed by the primer used in the cDNA amplification by means of PCR"

(ix) FEATURE:
(B) LOCATION: 301..324
(D) OTHER INFORMATION: /note= "This sequence was imposed by the primer used in the cDNA amplification by means of PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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 1               5                  10                  15

GAT AGC GTC AGT CTT TCC TGT AGG GCC AGC CAA AGT ATT AGC AAC AGC          96
Asp Ser Val Ser Leu Ser Cys Arg Ala Ser Gln Ser Ile Ser Asn Ser
            20                  25                  30

CTA CAC TGG TAT CAA CAA AAA TCA CAT GAG TCT CCA AGG CTT CTC ATC         144
Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile
        35                  40                  45

AAG TAT GTT TCC CAG TCC ATC TCT GGG ATC CCC TCC AGG TTC AGT GGC         192
Lys Tyr Val Ser Gln Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

AGT GGA TCA GGG ACA GAT TTC ACT CTC ACT ATC AAC AGT GTG GAG ACT         240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Asn Ser Val Glu Thr
65                  70                  75                  80

AAA GAT TTT GGA ATG TAT TTC TGT CAA CAG AGT GAC AGT TGG CAG TGG         288
Lys Asp Phe Gly Met Tyr Phe Cys Gln Gln Ser Asp Ser Trp Gln Trp
                85                  90                  95

ACG TTC GGT GGA GGG ACC AAG CTG GAG ATC AAA CGT                         324
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Thr Pro Gly
 1               5                  10                  15

Asp Ser Val Ser Leu Ser Cys Arg Ala Ser Gln Ser Ile Ser Asn Ser
            20                  25                  30

Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile
        35                  40                  45

Lys Tyr Val Ser Gln Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Asn Ser Val Glu Thr
65                  70                  75                  80

Lys Asp Phe Gly Met Tyr Phe Cys Gln Gln Ser Asp Ser Trp Gln Trp
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE : N-terminal

(ix) FEATURE: Xaa rapresents an amino acid not exactly characterized

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Asp Ile Val Leu Thr Gln Ser Xaa Ala Thr Leu Ser Val Thr Pro Gly
1               5                  10                  15

Asp Ser Val Ser Leu Xaa Xaa Arg Ala Ser Gln Ser
            20                  25
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ANTI-SENSE: NO

(ix) FEATURE: 5' primer 5' specific for the leader sequence of light ang heavy chains for use in PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CACAGGTCTA GACCATGGGA TGGAGCTGTA TC 32

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ANTI-SENSE: NO

(ix) FEATURE: CKhu primer specific for the constant region of the light chain for use in PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTAACTCCCG GGTTAACACT CTCCCCTGTT GAAGCTCTT 38

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: singol
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ANTI-SENSE: YES

(ix) FEATURE: VH1FOR anti-sense primer specific for the sequence of a fragment of the constant region of the heavy chain for use in PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTGGGGCCAA GGGACCACGG TCACCGTCTC CTCA 34

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) CHAIN: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ANTI-SENSE: NO

(ix) FEATURE: CHhu primer specific for the sequence at the 3' end of a fragment of the constant regiion of the heavy region for use in PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTAACTCCCG GGTTATCCCG GAGACAGGGA GAGGCT 36

Claims

1. Murine/human chimeric monoclonal antibody, termed chMint5, or a fragment thereof specific for the EGF receptor (EGF-R).

2. Monoclonal chMint5 antibody or a fragment thereof, according to claim 1, characterized in that it comprises the variable regions of the heavy (VH) and light (VL) chains of the monoclonal antibody produced by DSM ACC2150 hybridoma.

3. Monoclonal chMint5 antibody or a fragment thereof, according to claim 1, characterized in that it comprises the variable regions of the heavy (VH) and light (VL) chains of the monoclonal antibody produced by DSM ACC2150 hybridoma and the constant regions of the heavy and light chains of a human immunoglobulin or a fragment thereof.

4. Monoclonal chMint5 antibody or a fragment thereof, according to claim 3, comprising Cgamma1 and CK regions of human immunoglobulins or fragments thereof.

5. Monoclonal chMint5 antibody or a fragment thereof, according to claims 2-4, comprising the amino acid sequences indicated in SEQ ID NO:2 and SEQ ID NO:5 sequences.

6. Nucleotide sequences, indicated in SEQ ID NO:1 and SEQ ID NO:4, coding for the variable regions of the heavy (VH) and light (VL) chains of the chMint5 antibody described in claims 1-5.

7. Amino acid sequences indicated in SEQ ID NO:2 and SEQ ID NO:5.

8. Amino acid sequence indicated in SEQ ID NO:3.

9. Amino acid sequence indicated in SEQ ID NO:6.

10. Diagnostic method specific for the determination of the EGF-R, comprising the chMint5 antibody , according to claims 1-5 and a ligand

or a conjugate, optionally labeled or bound to a chromogen, which recognize the variable regions of said chMint5.

11. Diagnostic method, according to claim 10, wherein said ligand or conjugate comprises a sequence complementary to at least one of the sequences indicated in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6 or to fragments thereof.

12. Use of the chMint5 antibody, according to claims 1-5 in a diagnostic method according to claims 10-11.

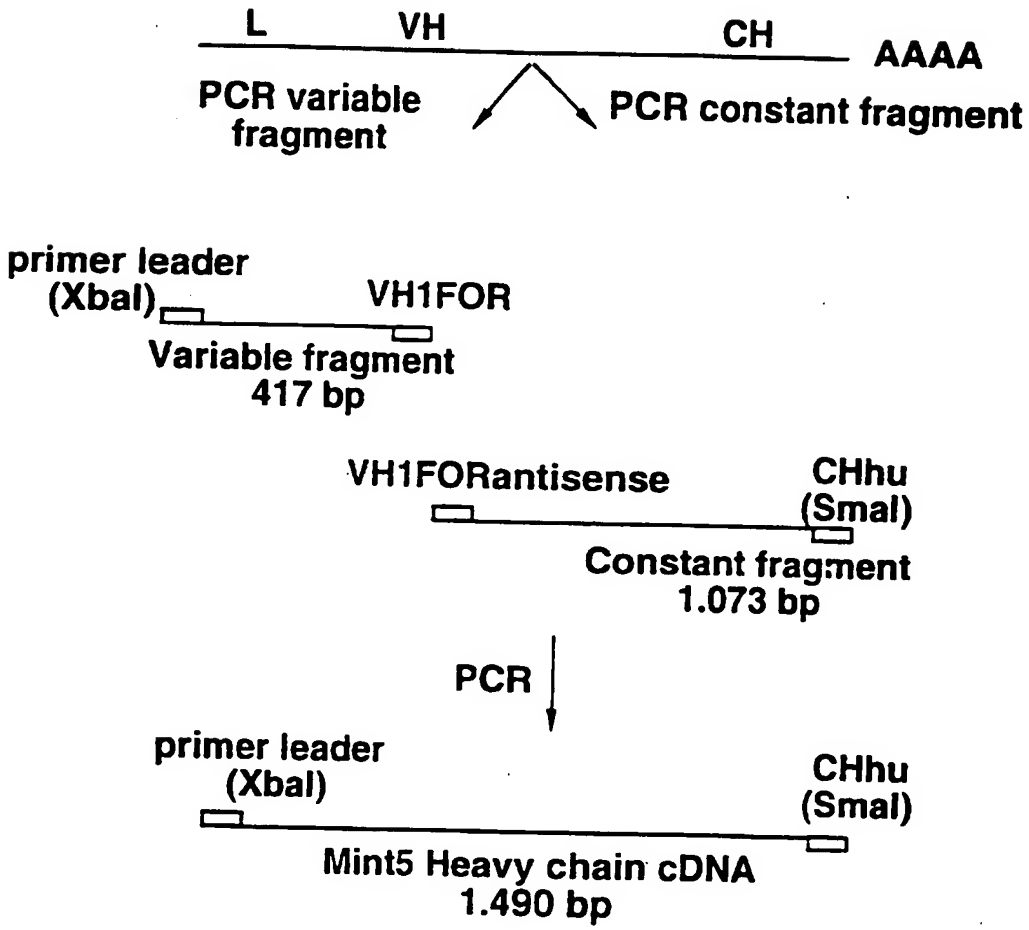
13. Use of the chMint5 antibody, according to claims 1-5, for the preparation of a pharmaceutical composition, useful in therapy, comprising a pharmaceutically acceptable carrier and/or excipient.

14. Immunotoxin or immunocytokin comprising the chMint5 antibody, according to claims 1-5, and one toxin.

15. Immunotoxin or immunocytokin, according to claim 14, obtained by means of chemical conjugation or by recombination of genes which correspond to chMint5 or to parts of it and by recombination of genes coding for a toxin.

16. Use of the immunotoxin or immunocytokin, according to claims 14-15, for the preparation of pharmaceutical compositions useful in the antitumor therapy, combined to a pharmaceutically acceptable carrier and/or excipient.

Figure 1


L
VH
CH
AAAA
PCR variable fragment
PCR constant fragment
primer leader (Xbal)
VH1FOR
Variable fragment 417 bp
VH1FORantisense
CHhu (Smal)
Constant fragment 1.073 bp
PCR
primer leader (Xbal)
CHhu (Smal)
Mint5 Heavy chain cDNA 1.490 bp

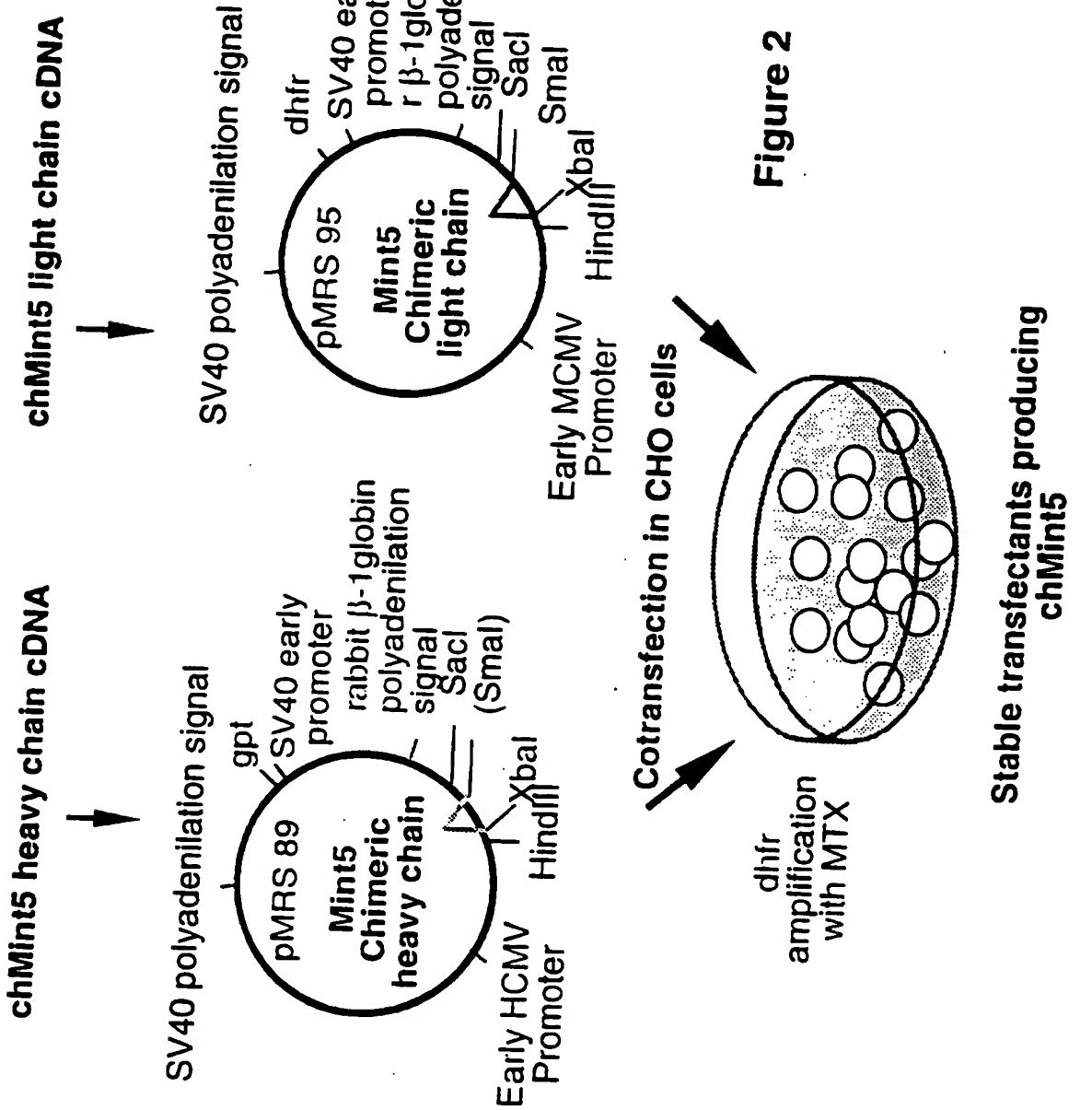

chMint5 heavy chain cDNA
chMint5 light chain cDNA
SV40 polyadenilation signal
gpt
SV40 early promoter
rabbit β-1globin polyadenilation signal
SacI (SmaI)
XbaI
HindIII
pMRS 89
Mint5 Chimeric heavy chain
Early HCMV Promoter
SV40 polyadenilation signal
dhfr
SV40 early promoter
r β-1globin polyadenil. signal
SacI
SmaI
XbaI
HindIII
pMRS 95
Mint5 Chimeric light chain
Early MCMV Promoter
Cotransfection in CHO cells
dhfr amplification with MTX
Stable transfectants producing chMint5

Figure 2

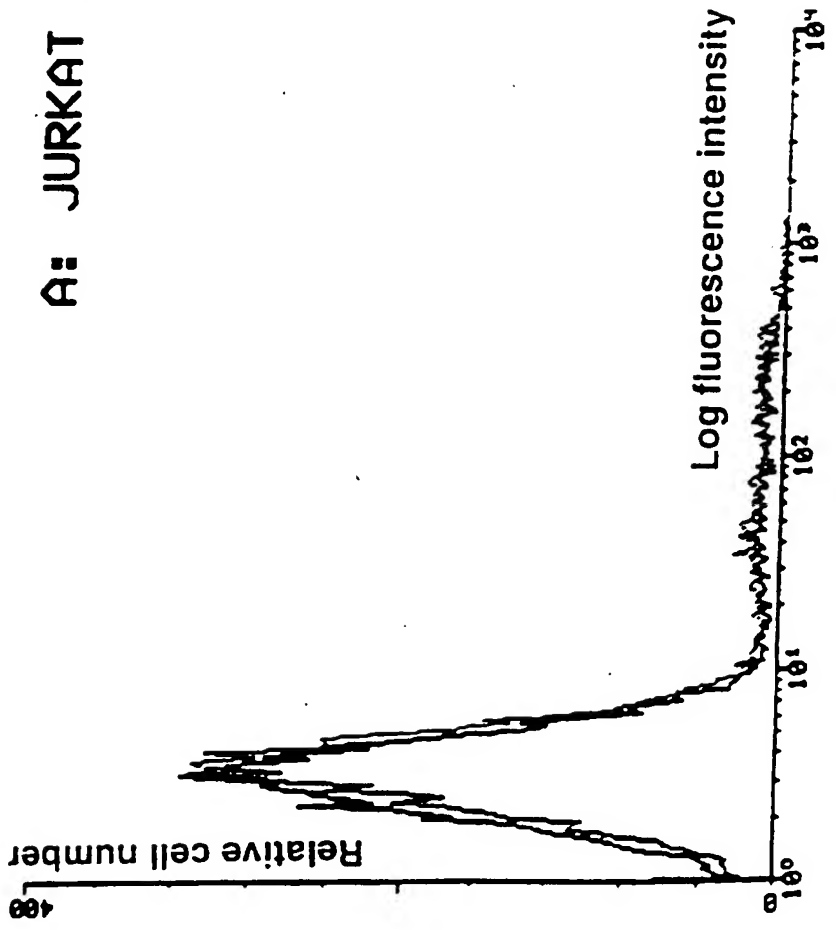

A: JURKAT
Log fluorescence intensity
Relative cell number
0
100
10¹
10²
10³
10⁴
400

Figure 3A

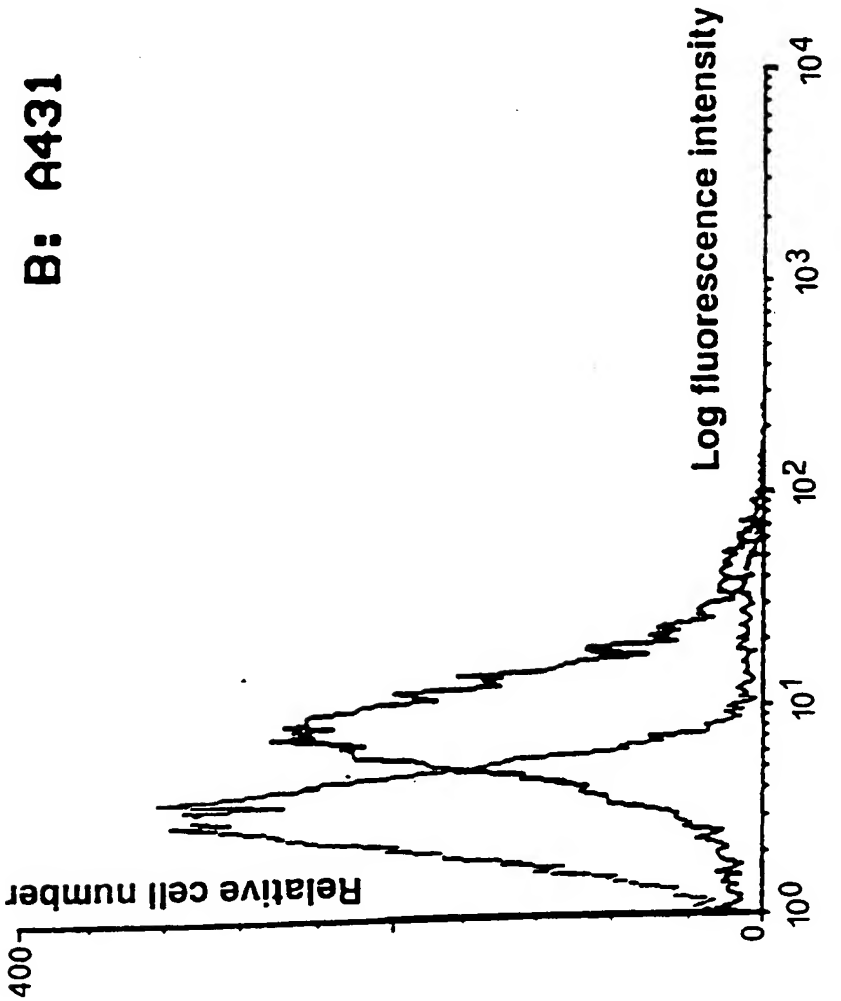
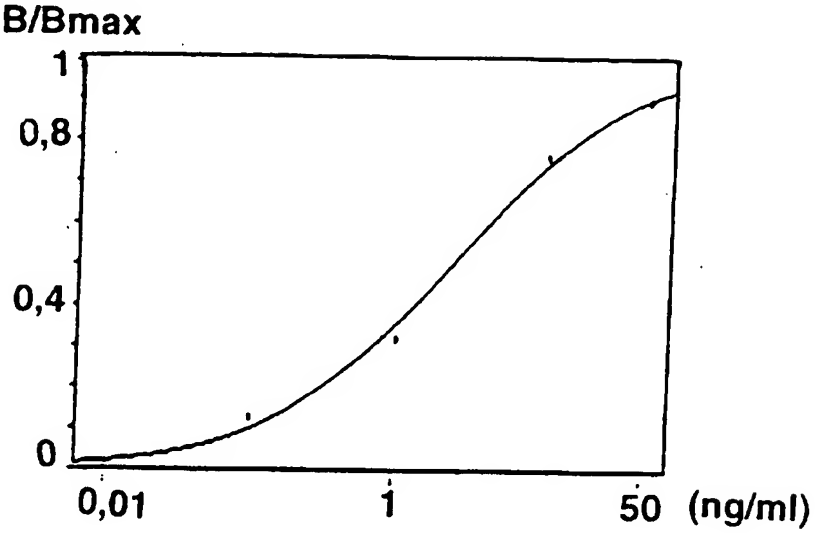

B: A431
Relative cell number
400
0
Log fluorescence intensity
10^0
10^1
10^2
10^3
10^4

Figure 3B

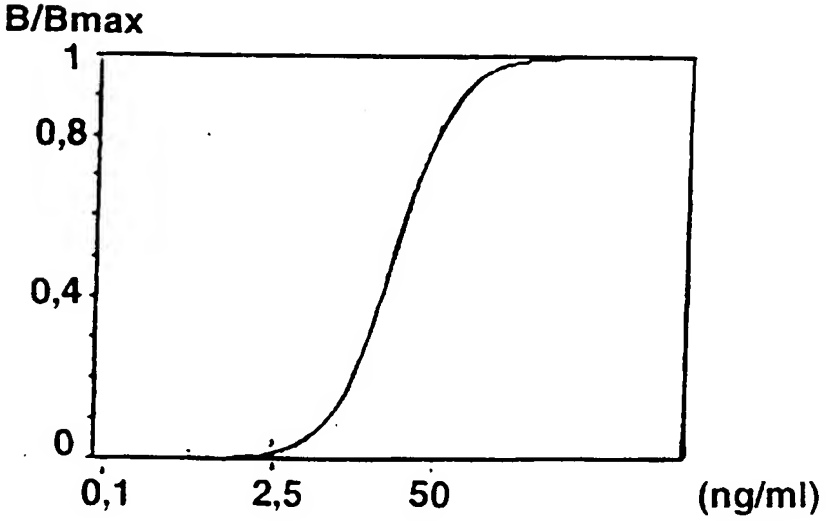
Figure 4

A



Affinity constant= 2,05E+10L/mol

B



Affinity constant= 6,76E+09L/mol

INTERNATIONAL SEARCH REPORT

Intern ul Application No
PCT/EP 96/00805

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/28 C07K16/46 G01N33/577 G01N33/68
A61K39/395 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 15683 (MERCK GMBH) 17 September 1992 see page 30, line 1 - page 33, line 18 see claims see example 2 ---	1-15
Y	EUROPEAN JOURNAL OF CANCER, vol. 27, no. suppl. 3, 1991, OXFORD, GB, page S57 XP002003433 E. TOSI ET AL.: "The anti-EGFR Mint5 mAb is able to specifically target RIP alpha-sarcin cytotoxicity against relevant target cells." see abstract 7.041 --- -/--	1-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 1996	17. 06. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Inter ual Application No
PCT/EP 96/00805

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 4, April 1994, WEINHEIM, GERMANY, pages 952-958, XP000570464 C. KETTLEBOROUGH ET AL.: "Isolation of tumor cell-specific single-chain Fv from immunized mice using phage-antibody libraries and the re-construction of whole antibodies from these antibodies fragments." see page 956, left-hand column, line 21 - line 58 see figure 4 ---	1-15
Y	EUROPEAN JOURNAL OF CANCER, vol. 27, no. suppl. 3, 1991, OXFORD, GB, page S82 XP000570465 S. MUNOZ ET AL.: "Biochemical and toxic properties of an immunotoxin composed of an anti-EGFR mouse mAb and the RIP alpha-sarcin." see abstract 11.067 ---	1-15
A	PROTEIN ENGINEERING, vol. 4, no. 7, October 1991, OXFORD, GB, pages 773-783, XP002003434 C. KETTLEBOROUGH ET AL.: "Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation." see the whole document ---	1-15
A	CANCER IMMUNOLOG IMMUNOTHERAPY, vol. 37, no. 5, 1993, HEIDELBERG, GERMANY, pages 343-349, XP000570462 M. NARAMURA ET AL.: "Therapeutic potential of chimeric and murine anti-(epidermal growth factor receptor) antibodies in a metastasis model for human melanoma." see the whole document ---	1-15
A	BREAST CANCER RESEARCH AND TREATMENT, vol. 29, no. 1, January 1994, THE HAGUE, NL, pages 127-138, XP000570461 J. BASELGA ET AL.: "The epidermal growth factor receptor as a target for therapy in breast carcinoma." see page 135, left-hand column ---	1-15

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1

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/00805

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH. ANNUAL MEETING., vol. 33, 1992, BALTIMORE, MD, USA, page 340 XP000570445 B. MUELLER ET AL.: "Effect of a chimeric anti-EGF receptor antibody on melanoma metastasis." see abstract 2026 ---	1-15
A	CANCER IMMUNOLOGY IMMUNOTHERAPY, vol. 34, no. 1, 1991, HEIDELBERG, GERMANY, pages 37-42, XP000570463 R. PELLEGRINI ET AL.: "Characterization of a monoclonal antibody directed against the epidermal growth factor receptor binding site." see the whole document ---	1-15
A	EP,A,0 586 002 (CENTRO DE IMMUNOLOGIA MOLECULAR) 9 March 1994 see page 4, line 45 - line 55 see page 7, line 58 - page 8, line 5 ---	1-15
P,X	WO,A,95 07297 (MENARINI RICERCHE SUD S.P.A.) 16 March 1995 see the whole document -----	1-15

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

I ·national application No.

PCT/EP 96/00805

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10-12
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims (partially, as far as in vivo methods is concerned) are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/00805

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9215683	17-09-92	AU-B-	658396	13-04-95
		AU-B-	1340392	06-10-92
		CA-A-	2082160	07-09-92
		CZ-A-	9203327	16-02-94
		EP-A-	0531472	17-03-93
		HU-A-	65687	28-07-94
EP-A-586002	09-03-94	CA-A-	2116753	02-09-94
		CN-A-	1103404	07-06-95
WO-A-9507297	16-03-95	AU-B-	7694594	27-03-95